Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis mice

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Introduction

The genetic disorder cystic fibrosis (CF), which affects approximately 80,000 individuals in Europe and North America, is caused by mutations in the CF Transmembrane Conductance Regulator (*CFTR*) gene[1-3]. Chronic microbial lung infections, most commonly caused by the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, reduce the life expectancy of CF patients due to excessive lung tissue remodelling and destruction[4,5]. How mutation or absence of CFTR, which is primarily expressed in ciliated and submucosal gland epithelial cells of the respiratory tract[6,7], promotes pulmonary infections, is still incompletely understood.

Several studies support the notion that CF cells and respiratory tissues exhibit a pro-inflammatory status, which may facilitate bacterial lung colonization and infection. Increased NFκB and interleukin (IL)-8 expression was demonstrated in CF versus control cells[8,9]. Furthermore, even non-infected CF mice showed signs of inflammation[10]. Finally, markers of inflammation were significantly elevated in lungs from aborted embryos with CF and in bronchoalveolar lavage fluids from CF patients as young as four weeks, with negative cultures for CF-related bacteria, virus and fungi[11,12].

In the present study we investigated the role of sphingolipids in the pathogenesis of CF. Cftr belongs to the ATP binding cassette (ABC)-transporter family, which has been previously demonstrated to be involved in lipid transport[13-15]. Furthermore, two studies indicate that defective Cftr leads to higher pH levels in intracellular organelles[16,17], which might be important for the regulation of the cellular sphingolipid metabolism by enzymes with an activity peak at acidic pH values. Since sphingolipids are critically involved in

the regulation of cell survival[18,19], NFκB activation[20] and expression of pro-inflammatory cytokines[21], we hypothesized that an alteration of the sphingolipid metabolism may play an important role in the pathogenesis of CF lung disease.

Here, we demonstrate that deficiency of functional Cftr results in an accumulation of cellular ceramide in the respiratory tract of uninfected CF-mice. The accumulation was caused by the alkalinization of Cftr-deficient vesicles in respiratory cells leading to an imbalance of the activities of the acid sphingomyelinase (Asm) and acid ceramidase (Ac). A similar accumulation of ceramide was detected in respiratory epithelial cells and airways of CF patients. In CF-mice, the increased sphingolipid concentrations triggered chronic pulmonary inflammation, death of respiratory epithelial cells and deposition of DNA in bronchi, and resulted in high susceptibility to severe pulmonary *P. aeruginosa* infections. Normalization of pulmonary ceramide levels that was achieved by pharmacological and genetic blockade of Asm, prevented all pathological findings and, most importantly, protected CF-mice from severe pulmonary *P. aeruginosa* infections.

Results

Cftr-deficiency results in ceramide and sphingosine accumulation in the respiratory tract

To test the hypothesis that deficiency of functional Cftr results in alterations of the sphingolipid metabolism in the respiratory tract, we determined the concentration of ceramide in the lung of two wild type mouse strains and in the mouse strains $Cftr^{tm1Unc}$-$Tg^{(FABPCFTR)}$ (abbreviated $Cftr^{KO}$) and B6.129P2(CF/3)-$Cftr^{TgH(neoim)Hgu}$ (abbreviated $Cftr^{MHH}$, syngenic to C57BL/6). These mice lack functional *Cftr* in the respiratory tract (collectively named CF-mice). Our results demonstrate a significant increase of ceramide in lungs of mice deficient for functional Cftr compared to control animals (Fig. 1a). Importantly, this increase was age-dependent, whereas ceramide levels did not differ with age in wild type mice.

To identify cells accumulating ceramide in $Cftr^{KO}$ and $Cftr^{MHH}$ mice, we isolated bronchial epithelial cells and stained the cells with a Cy3-coupled monoclonal anti-ceramide antibody (clone MAS 0010). Excessive accumulation of ceramide was observed in ciliated respiratory cells of CF-mice but not wild-type mice (Fig. 1b). These data were confirmed employing a different anti-ceramide antibody (clone 15B4), which revealed a similar accumulation of ceramide in respiratory epithelial cells from CF-mice (not shown). To further locate ceramide over-expressing cells, we stained paraffin sections of lungs from wild-type, $Cftr^{KO}$ and $Cftr^{MHH}$ mice for ceramide. Ceramide accumulation was observed in the respiratory tract epithelium and the submucosa of CF-mice (Fig. 1c). Alveolar epithelial cells that constitute the vast majority of cells in the lung, did not

contain significant amounts of ceramide, neither in wild-type nor in *Cftr*^{KO} and *Cftr*^{MHH} mice (Fig. 1c). Concurrent with the presence of Cftr in macrophages[17], we detected a 6-fold increase of ceramide in murine lung macrophages obtained by pulmonary lavage, further supporting our notion that a defect in Cftr results in ceramide accumulation (data not shown).

To address the topology of ceramide in respiratory epithelial cells, we performed confocal microscopy. These studies indicate a marked accumulation of ceramide-containing vesicles in respiratory epithelial cells of *Cftr*-deficient mice, and also the accumulation of ceramide in other cell membranes (Fig. 1d). FACS data confirm the accumulation of ceramide-containing vesicles in lung extracts from *Cftr*-deficient mice (Fig. 1e) as indicated by the increased percentage of ceramide-positive vesicles. In addition, we detected an increase of the absolute ceramide amount in these vesicles as indicated by the increased Cy3-signal that measures binding of the Cy3-labelled anti-ceramide antibodies in the FACS studies. The FACS-data demonstrate that the accumulation of ceramide-containing lysosomes already occurs in young, 12 week old *Cftr*-deficient mice, but to a lower degree than in older mice. This suggests that the accumulation of ceramide in *Cftr*-deficient mice originates in vesicles.

To further support the accumulation of ceramide in *Cftr*-deficient cells and to exactly localize ceramide, we performed immunogold electron microscopy on lung sections from wild-type and *Cftr*-deficient mice. Although immunogold electron microscopy studies can only provide semi-quantitative results, they support the accumulation of ceramide in *Cftr*-deficient respiratory epithelial cells, observed in our FACS and confocal microscopy studies (Fig. 1f). Ceramide predominantly locates to intracellular vesicles in *Cftr*-

deficient respiratory epithelial cells versus normal control cells (Fig. 1f). The accumulation of ceramide was not restricted to cathepsin D positive lysosomes, but also observed in other vesicles.

To test whether these findings in CF mouse strains are also relevant for CF patients, we isolated nasal respiratory epithelial cells from adult CF patients and healthy individuals and stained the cells for ceramide (monoclonal antibodies MAS 0010 and 15B4). Similarly, lung transplant material from three CF patients and lung material from three normal donors was stained. The results show an accumulation of ceramide in the membrane of nasal epithelial cells (Fig. 1g), respiratory epithelial cells (Fig. 1h) and submucosal glands (Fig. 1i) compared to the respective cells or tissues from healthy individuals. Quantification of the fluorescence in epithelial cells from 17 healthy and 18 CF individuals reveals a 4 ± 0.4-fold increase of the ceramide signal in cells from CF-patients ($p < 0.01$, t-test).

Taken together, these data indicate that deficiency of functional Cftr results in an age-dependent accumulation of ceramide in murine epithelial and submucosal cells of the respiratory tract and strongly suggest that a similar accumulation also occurs in CF patients.

Ceramide accumulation is a consequence of defective acidification of intracellular vesicles in cells expressing defective Cftr

Recent studies by Di et al.[17] showed that CFTR-deficiency in alveolar macrophages results in a lysosomal pH shift from pH 4.5 to at least pH 5.9. Studies by He et al.[22] and

Spence et al.[23] demonstrated that an increase of the pH to 5.9 reduces the Asm activity by only ~35%, while the activity of the Ac is reduced by more than 90% at this pH, in some respects mimicking Farber disease, which is caused by a deficiency of the Ac and results in an accumulation of ceramide. Furthermore, at a pH of 5.9, the Ac was shown to have a reverse activity producing ceramide instead of consuming it[22]. The imbalance of the Asm and the Ac and the reverse activity of the Ac at a vesicular pH of 5.9 should result in a net accumulation of ceramide in an *in vivo* situation.

To investigate this hypothesis, we determined the pH in cellular vesicles of respiratory epithelial cells from wild-type and 24-week old CF-mice. Measurements of the vesicular pH in *Cftr*-deficient respiratory epithelial cells employing lysosensor-green revealed an increase of the pH in these vesicles to a value of 5.9 (Fig. 2a). This increase was mimicked by adding the Cftr-inhibitor 172 to wild-type cells (Fig. 2a).

Next, we confirmed the pH dependency of Asm and Ac activity in murine lung preparations (Fig. 2b) as well as the reverse activity of the Ac at ph 4.5, 5.0 and pH 5.9 (Fig. 2b). The data show an almost complete inhibition of the Ac at pH 5.9. In fact, at this pH, the enzyme showed a reverse activity producing ceramide. In contrast, the activity of the Asm was reduced by only 35% at pH 5.9.

To show that an inhibition of the Ac results in cellular ceramide accumulation, we inhibited the Ac in respiratory epithelial cells of wild type mice *in vivo* by inhalation of n-oleoylethanolamine. Alternatively, intracellular vesicles were alkalinized by inhalation of bafilomycin, nigericin, chloroquin or NH_4Cl. The results confirm that direct inhibition of the Ac or alkalinization of lysosomes results in increased concentrations of ceramide in wild-type respiratory cells (Fig. 2c).

To directly demonstrate a critical role of the lysosomal pH for the accumulation of ceramide in *Cftr*-deficient cells, we acidified isolated vesicles from *Cftr*-deficient lung cells *in vitro*. In the FACS studies on isolated vesicles from *Cftr*-deficient lung cells that were either left at a pH of 5.9 or acidified to pH 4.5 (lower panels in Fig. 2d) the intensity of the Cy3-anti-ceramide staining served to measure the amount of ceramide in a single vesicle. The data demonstrate that acidification of vesicles isolated from *Cftr*-deficient cells reduces the vesicular ceramide amount (Fig. 2d). Vice versa, alkalinisation of vesicles isolated from wild-type cells from pH 4.5 to pH 5.9 resulted in ceramide-accumulation. Taken together, these data indicate that ceramide accumulation is a consequence of defective acidification of intracellular vesicles in cells lacking functional Cftr.

Previous studies demonstrated that Cftr also functions as a direct transmembrane transporter and/or regulator of the transmembrane transport of sphingosine-1-phosphate[15]. However, we did not detect a significant increase of sphingosine-1-phosphate levels (2.6 ± 1.1 pmol/mg protein in $Cftr^{MHH}$ compared to 2.8 ± 1.1 pmol/mg protein in wild type mice). Sphingosine was increased in lungs of CF-mice (4.08 ± 0.25 pmol/mg protein in $Cftr^{MHH}$ compared to 1.0 ± 0.11 pmol/mg protein in wild type mice), but the total sphingosine amounts were ~2000-fold lower than those of ceramide. This suggests that a defect in uptake of sphingosine-1-phosphate via Cftr might not be the major cause for ceramide accumulation in *Cftr*-deficient cells. Nevertheless, we tested whether the uptake of [^{3}H]sphingosine and [^{3}H]sphingosine-1-phosphate is affected by *Cftr*-deficiency in tracheal epithelial cells. More than 95% of the added [^{3}H]sphingosine were converted to [^{3}H]sphingosine-1-phosphate within 5 min after addition to tracheal cells from wild type,

Cftr^{KO} and *Cftr*^{MHH} mice (not shown). The uptake of [^{3}H]sphingosine-1-phosphate was reduced by approximately 50% (not shown) in *Cftr*-deficient epithelial cells. Thus, a transport defect of sphingosine-1-phosphate might contribute to a further change of sphingolipids, in particular sphingosine, in *Cftr*-deficient cells.

Inhibition of the Asm normalizes ceramide in the respiratory tract of *Cftr*-deficient mice

Ceramide is generated in cellular membranes from sphingomyelin by the constitutive activity of Asm or by de-novo synthesis[24-26]. The data described above indicate that the accumulation of ceramide within the lung of *Cftr*-deficient mice is caused by an imbalance between Asm and Ac activities. Thus, the Asm might be a potential pharmacological target to manipulate the sphingolipid metabolism in CF-mice. We, therefore, tested whether inhibition of Asm normalizes the increased ceramide concentration in the respiratory tract of *Cftr*^{KO} and *Cftr*^{MHH} mice. Partial inhibition of Asm was achieved by intraperitoneal injection of amitriptyline (10 mg/kg twice daily for 2.5 days), which induces a proteolytic degradation of Asm[27] or by crossing *Cftr*^{KO} mice with Asm knock-out mice (*Smpd1*$^{-/-}$) to obtain mice deficient for Cftr and heterozygous for Asm (*Cftr*^{KO}/*Smpd1*$^{+/-}$) (Fig. 2e). Biochemical assays and immunofluorescence studies on respiratory epithelial cells from *Cftr*^{KO}, *Cftr*^{MHH} and *Cftr*^{KO}/*Smpd1*$^{+/-}$ mice revealed that amitriptyline-mediated degradation or heterozygosity of Asm almost normalized pulmonary ceramide levels in these mice (Figs. 2f and 2g).

Amitriptyline has been recently shown to result in degradation of the Ac in addition to the Asm[28]. Our data indicate that amitriptyline in fact also reduced the already very

low Ac activity at pH 5.9, but also the ceramide-producing reverse activity of the Ac at this pH (Fig. 2e). Thus, inhibition of ceramide generation via Asm and Ac is the predominant effect of amitriptyline at pH 5.9 resulting in net-reduction of cellular ceramide. Since the specific activity of the Asm is approximately 40-fold higher than the reverse activity of the Ac and 250-fold higher than the ceramide-consuming activity of the Ac at a pH of 5.9, we assume the Asm as the primary target of amitriptyline. This is also consistent with the observation that heterozygosity of the Asm, which does not affect Ac expression, also reduced ceramide levels in CF mouse lungs.

The data indicate that deficiency of *Cftr* results in an accumulation of ceramide in the respiratory tract that is corrected by pharmacological or genetic inhibition of Asm and Ac.

Normalization of pulmonary ceramide prevents infection of CF-mice with *P. aeruginosa*

To test the significance of these findings for bacterial lung infections in CF, we determined the susceptibility of wild-type, $Cftr^{KO}$ and $Cftr^{MHH}$ mice to develop pulmonary *P. aeruginosa* infections. Increased susceptibility of CF mice for *P. aeruginosa* lung infection has been reported previously[29-31]. Mice were intranasally inoculated with 10^8 CFU (planctonic, early log phase grown) of the clinical *P. aeruginosa* isolates 762 and 769 or the laboratory strain ATCC 14115[21]. While wild-type murine lungs contained only $\sim 10^3$ CFU of *P. aeruginosa* per 100 milligram of homogenized lung tissue 2 h after inoculation, $Cftr^{KO}$ and $Cftr^{MHH}$ mice were significantly more susceptible to infection, revealing $\sim 10^6$ CFU of *P. aeruginosa* strain 762/100 mg lung tissue (Fig. 3a).

Pharmacological or genetic inhibition of Asm protected *Cftr*^{KO} and *Cftr*^{MHH} mice from pulmonary *P. aeruginosa* infections. Amitriptyline-treated *Cftr*^{KO} or *Cftr*^{MHH} mice or *Cftr*^{KO}/*Smpd1*$^{+/-}$ mice, respectively, displayed substantially less *P. aeruginosa* CFU in the lung than the respective CF-mouse strains 2 h after intranasal inoculation with 10^8 CFU of *P. aeruginosa* 762 (Fig. 3a). Similar data were obtained after infection of *Cftr*^{KO} or *Cftr*^{MHH} mice with *P. aeruginosa* strains 769 and ATCC 14115 (Fig. 3b). Importantly, the susceptibility of *Cftr*^{KO} and *Cftr*^{MHH} mice to develop pulmonary *P. aeruginosa* infections increased with the age of the mice (Fig. 3a), corresponding to the age-dependent increase of pulmonary ceramide concentrations. A slightly increased susceptibility to bacterial infection was already detectable in CF animals at an age of 8 and 12 weeks (Fig. 3a), although the susceptibility of younger mice was much lower than in mice with 16 and 24 weeks of age consistent with the lower accumulation of ceramide in young mice lung´s.

Control experiments revealed that when mice were intranasally challenged with 10^8 CFU of planctonic (early log phase) *P. aeruginosa*, almost all bacteria could be recovered from the noses of the mice 2 min after application, whereas the lungs were free of pathogens (Fig. 3c). Importantly, there was no difference in recovered CFUs between wild-type and CF-mice at this early time point. In the early phase, i.e. 15, 30 and 60 min after infection, *P. aeruginosa* migrated to the lower airways and bacterial numbers increased in the lungs of wild-type, CF-, amitriptyline-treated or *Cftr*$^{-/-}$/*Smpd1*$^{+/-}$-CF-mice to a similar degree (Fig. 3c). After approximately 60 min wild-type mice started to eliminate the bacteria, whereas in CF-mice bacteria started to grow exponentially, consistent with the notion that these mice are highly susceptible to infection with *P. aeruginosa*. Infection of the mice with 10^7 CFU resulted in approximately 10-fold lower

absolute numbers in the lung 2 h after the infection, revealing again a significant difference in CFUs between wild-type and CF-mice as observed for the higher infections dose (Fig. 3c).

We also challenged the lungs of *Cftr*-deficient and wild-type mice with *Streptococcus pneumoniae*, a pathogen, which rarely infects CF patients. The data revealed no difference between the pulmonary infection of wild-type and CF-mice with *S. pneumoniae* (not shown) suggesting that the release of DNA in the airways of *Cftr*-deficient mice provides at least some specificity for an infection with *P. aeruginosa.*

Next, we explored the relation of *P. aeruginosa* lung colonization rates to mouse mortality. The analysis of the 7 d survival rates demonstrated that initially high *P. aeruginosa* CFU correlated with mortality, which was significantly reduced by pharmacological or genetic heterozygosity of Asm (Fig. 3d).

In summary, these results indicate that the observed ceramide accumulation is critical for bacterial infection in CF-mice.

Accumulated ceramide cause inflammation in the lungs of CF-mice

Next, we aimed to identify mechanisms how accumulated ceramide mediates the hypersusceptibility of CF-mice to develop *P. aeruginosa* infections. To this end we tested typical immunological changes in the respiratory tract that are known to increase the susceptibility to *P. aeruginosa* and/or have been demonstrated to be altered in CF cells or lung tissues. Those changes include neutrophil/macrophage recruitment and altered cytokine expressions[8-12]. First, we determined the pulmonary concentrations of pro-inflammatory mediators in the lung of CF and wild type mice. $Cftr^{KO}$ or $Cftr^{MHH}$ mice

exhibited a constitutive increase of IL-1 and keratinocyte-derived chemokine (KC), the murine homologue of human IL-8 in their lungs (Fig. 4a). Genetic or pharmacological inhibition of Asm normalized levels of pulmonary IL-1 and KC/IL-8 in CF mice (Fig. 4a), suggesting that cytokine up-regulation is linked to ceramide accumulation in CF-mice. Importantly, the mice, held in a pathogen-free environment, were free of any infection as defined by repeated microbial cultures of lung homogenates and serological testing for multiple microbial species (for details see Methods).

In addition to the age-dependent increases in ceramide, IL-1 and IL-8 expression, lung tissues of CF-mice exhibited an age-dependent increase of macrophage and neutrophil cell numbers that was corrected by genetic or pharmacological inhibition of Asm (Figs. 4b and c). Compared to wild-type mice, $Cftr^{KO}$ and $Cftr^{MHH}$ mice exhibited significantly increased macrophage (Fig. 4b) and neutrophil (Fig. 4c) cell counts in the lung at an age of 30 and 52 weeks. The high macrophage and neutrophil cell numbers were normalized in $Cftr^{KO}/Smpd1^{+/-}$ mice and upon treatment of $Cftr^{KO}$ mice with amitriptyline (Figs. 4b and c). In $Cftr^{KO}$ mice, macrophages (Figs. 4d-g) and neutrophils (Fig. 4j, k) clustered around murine submucosal glands (Fig. 4e), the principal site of CFTR expression in humans[6], and in the bronchial associated lymphoic tissue (BALT) (Figs. 4f, g), while only marginal numbers of macrophages (Figs. 4h and i) and neutrophils (Figs. 4l, m) were present in the lungs of wild-type mice. These findings suggest that ceramide accumulation is linked to lung inflammation in uninfected CF-mice, which may facilitate bacterial lung infection.

Accumulated ceramide mediates a constitutive increase of respiratory cell death and deposition of DNA on the respiratory epithelium of CF-mice

Besides its impact on cytokine expression and inflammatory cell recruitment, ceramide has previously been shown to be critically involved in the induction of cell death[18,19]. Therefore, we tested whether CF-mice display an increased death rate of respiratory epithelial cells and whether this contributes to the hypersusceptibility of CF-mice to develop *P. aeruginosa* infections.

TUNEL staining of lung sections of $Cftr^{KO}$ or $Cftr^{MHH}$ mice revealed a higher number of dead cells, dispersed in the respiratory mucosa of large and medium-sized bronchi when compared to wild type mice, in which cell death was rarely observed (Figs. 5a and b). The rate of respiratory epithelial cell death was increased in CF-mice in an age-dependent manner starting with a few dead cells in the respiratory tract in 12 week old mice (Fig. 5b). The increased death rate of respiratory epithelial cells in $Cftr^{KO}$ or $Cftr^{MHH}$ mice was normalized by pharmacological or genetic inhibition of Asm (Figs. 5a and b) or application of the broad-spectrum caspase inhibitor zVAD that has been previously shown to block apoptosis in vivo[32] (Figs. 5a and b).

Next, we investigated whether increased cell death, caused by the accumulation of ceramide in nasal and bronchial epithelial cells, results in DNA deposits on the respiratory epithelium. DNA deposits were detectable on the mucosal lung surface in 12%-17% of bronchi in $Cftr^{KO}$ and $Cftr^{MHH}$ mice (Figs. 5c and d), compared to maximal 2% DNA deposits in wild type mice. Young, 12-week old mice displayed a small, but distinct increase of DNA deposits in bronchi (Fig. 5d). To test whether the increased cell death rate in CF-mice was linked to deposition of DNA in the airways of these mice, we

treated *Cftr*^{KO} and *Cftr*^{MHH} mice with the broad-spectrum caspase inhibitor zVAD. ZVAD inhibited the formation of DNA deposits within airway mucus in CF-mice (Figs. 5c and d). Importantly, pharmacological inhibition of Asm by amitriptyline, genetic heterozygosity of *Asm* or inhalation of recombinant DNase also abrogated DNA deposition on the mucosal lung surface (Figs. 5c and d).

These findings support the notion that ceramide accumulation increases the epithelial cell death rate resulting in abnormal deposition of DNA in bronchi of CF-mice.

DNA deposits on the respiratory epithelium of CF-mice mediate adhesion of and infection with *P. aeruginosa*

To investigate whether DNA deposits on the respiratory epithelium of CF-mice mediate adherence of *P. aeruginosa*, we first demonstrated that DNA, distributed on a carbohydrate matrix (agarose) significantly promotes adherence and growth of *P. aeruginosa* (Fig. 6a). Similarly, DNA distributed on a respiratory cell line led to an increased adherence of *P. aeruginosa* along DNA fibers (Fig. 6b) indicating the importance of DNA for bacterial adhesion. Therefore, we treated both *Cftr*^{KO} and *Cftr*^{MHH} mice with either recombinant human DNase (rhDNase) or with the broad-spectrum caspase inhibitor zVAD prior to bacterial challenge. Both, rhDNase or zVAD treatment effectively reduced *P. aeruginosa* cell numbers in lungs of *Cftr*^{KO} or *Cftr*^{MHH} mice (Fig. 6c), suggesting that DNA deposits derived from dead epithelial cells facilitate *P. aeruginosa* adhesion and infection. Neither rhDNase nor zVAD changed pulmonary ceramide levels (not shown).

Accumulation of ceramide in CF-mice is reduced by a diet

A recent study by Guilbault et al.[33] shows a decrease of ceramide in the lungs of *Cftr* knock out mice compared to their littermate controls, which seems to be inconsistent with our data. However, these studies employed mice that are completely deficient for *Cftr*. Mice completely lacking *Cftr* in the intestinum usually die after birth due to intestinal obstruction and therefore require a special liquid diet, for instance with Peptamen^R as in the study by Guilbault et al[33]. We tested whether this diet alters ceramide levels in the lungs of CF mice and fed our *Cftr*-deficient mice, which express a residual activity of *Cftr* (*Cftr*^{MHH} strain) or express *Cftr* in the intestinum as a transgen (*Cftr*^{KO} strain), with Peptamen^R. The data (Fig. 7a) demonstrate that Peptamen^R reduced ceramide levels in lungs of *Cftr*^{MHH} mice by 92% compared to untreated *Cftr*^{MHH} mice and in lungs of *Cftr*^{KO} mice by 86%. Compared to untreated wild-type mice, Peptamen^R-treated *Cftr*^{MHH} mice indeed revealed reduced ceramide levels as observed by Guilbault et al.[33]

To address the mechanisms that mediate the reduction of ceramide upon Peptamen^R feeding, we determined the concentration of cholesterol in the lungs of animals fed with Peptamen^R. Cholesterol has been previously shown to critically influence the activity of the Asm and high levels of cholesterol significantly reduce the activity of the Asm[34]. Our data demonstrate an ~ 3-fold increase of cholesterol in the lung upon Peptamen^R feeding (Fig. 7b). Simultaneous treatment of CF-mice with simvastatin (40 mg/kg/day i.p.), a known blocker of cholesterol[35] prevented the cholesterol accumulation (Fig. 7b) as well as the reduction of ceramide in the lungs of these animals (Fig. 7a). Consistent with its effect on ceramide, Peptamen^R diet resulted in ~60% inhibition of the activity of the Asm in the lung, an effect that was abrogated by treatment with simvastatin (Fig. 7c). These *in*

vivo data are supported by *in vitro* studies that show a dose-dependent inhibition of the Asm in isolated respiratory epithelial cells incubated with increasing doses of cholesterol (Fig. 7d).

Thus, we show that the chronic diet with Peptamen^{R} results in a marked accumulation of cholesterol that reduces Asm activity and pulmonary ceramide concentrations. These data explain the discrepancy between the two studies.

Discussion

In the present study we demonstrate an age-dependent hypersusceptibility of CF-mice to *P. aeruginosa* infection that is caused by increased cellular ceramide levels in the lungs of CF mice. Inhibition of Asm and/or Ac by treatment of $Cftr^{KO}$ or $Cftr^{MHH}$ mice with amitriptyline or by generation of $Cftr^{KO}/Smpd1^{+/-}$ mice normalized pulmonary ceramide levels, prevented pulmonary *P. aeruginosa* infections and increased survival of infected animals.

According to current models, sphingomyelin, present predominantly in the anti-cytoplasmic leaflet of cellular membranes[36], is constitutively metabolized to ceramide by Asm and further degraded to sphingosine by Ac[37]. This process is thought to be located predominantly in lysosomes but also in other membranes such as the plasma membrane. Our data indicate that acidification of intracellular vesicles mediated by Cftr presumably through the provision of counter ions to permit higher lumenal H^+ concentrations[17,38,39] is critical for the concerted regulation of ceramide by the activities of Asm and Ac. The alkalinization of Cftr-deficient vesicles in respiratory cells of CF mice to a pH of 5.9, results in an imbalance of the Asm and Ac activities with a reduced consumption of ceramide, finally resulting in a net accumulation of ceramide. Our FACS, confocal and electron microscopy studies indicate an increase in the number of ceramide-containing vesicles and increased ceramide concentrations within those vesicles and in the cell membranes of *Cftr*-deficient cells, predominantly in cells from older mice.

Two previous reports support our notion of defective acidification as a result of diminished Cl^- conductance in other CF cell types. Barasch et al.[16] demonstrated defective

acidification in the Golgi/trans-Golgi network, prelysosomes and endosomes of CF-cell lines leading to abnormal glycosylation of membrane proteins, and Di et al.[17] showed the same phenomenon in phagolysosomes of alveolar macrophages from *Cftr*-null mice leading to impaired bactericidal activity. These results have been questioned.[40-43] Methodological differences, particularly concerning the thiazolidinone CFTR inhibitor $CFTR_{inh}$-172, which is very sensitive to storage in stock DMSO solutions at low temperature, may account for some of the discrepant results between Di et al.[17] and Haggie and Verkman.[41] Independent of this controversy, it is difficult to compare data from Haggie and Verkman who investigated the acidification of phagosomes in wildtype and *Cftr*-deficient macrophages employing zymosan conjugates containing fluorescein and TMR, with our data determining the constitutive pH in vesicles of freshly isolated, non-cultured epithelial cells, employing lysosensor green.

A decrease of ceramide levels rather than an accumulation of ceramide was observed by other investigators in CF-mice, fed with the liquid diet Peptamen^{R}.[33] Here we demonstrate that Peptamen^{R} treatment results in a significant cellular accumulation of cholesterol that in turn results in reduction of Asm activity and pulmonary ceramide concentrations. CF-mice might be in particular sensitive to changes in the cholesterol metabolism,[44] since cultured *Cftr*-deficient cells already display 1.5-fold higher cholesterol levels than normal cells. These levels are too low to alter the activity of the Asm as demonstrated in the present study, however when CF mice are fed the Peptamen^{R} diet, cholesterol concentrations increase to such levels that Asm is severely affected. The increase in cholesterol concentrations is due to an impressive consumption of the diet by the mice (~75% of body weight in a 24-hour cycle) leading to liver steatosis.[45,46] It is

obvious that such a consumption is hard to achieve in humans, with the result that cholesterol levels will not increase in humans and, hence, Asm levels will not be changed. Further, any diet that requires a 3-4-fold increase of cellular cholesterol concentrations to be effective can not be used in humans due to the severe adverse effects of high cholesterol.

According to our data, showing a marked shift in ceramide and cholesterol levels, Peptamen^{R} may have also been responsible for the previously reported observed essential fatty acid imbalance in CF-mice.[47] This notion is supported by studies showing that rats, fed diets containing cholesterol, significantly increased the cellular level of arachidonic acid and the high arachidonic acid levels in hypercholesterolemic patients revealing high cellular cholesterol, relative to the protein content.[48-50]

We have identified two mutually non-exclusive pathways, which may facilitate bacterial lung infection in CF as a consequence of abnormal ceramide accumulation: (i) the increased release of dead cells into respiratory airways, resulting in the formation of DNA deposits, which may serve as bacterial adherence factors, and, (ii) an increased inflammatory response. The latter may negatively affect lung function, innate immune responses and change the airway architecture to allow enhanced bacterial adhesion, but also lung fibrosis.

While the link between ceramide and the induction of cell death has been extensively documented[18,19,51], conflicting results with regard to the rate of apoptosis in *Cftr*-deficient cells and mice prior[52] and after[53] *P. aeruginosa* infection have been previously described. Here, we show that an increased rate of cell death occurs only in the lungs of aged CF-mice, which was never investigated before. Increase of cell death is

most probably caused in cells with defective Cftr by accumulated ceramide, since Asm inhibition by amitriptyline or Asm heterozygosity in uninfected CF-mice reduced cell death to rates observed in wild type mice.

The increased rate of cell death causes DNA deposition on the airway epithelium of CF-mice, consistent with the observation that the broad-spectrum caspase inhibitor zVAD inhibited the formation of DNA/mucus deposits. Extracellular DNA has been previously shown to be required for the adhesion of *P. aeruginosa* to abiotic surfaces[54]. Our findings extend this notion and demonstrate that airway deposits of DNA are critical for the high susceptibility of CF-mice to develop *P. aeruginosa* infections, since rhDNase treatment rescued CF-mice from lethal *P. aeruginosa* infection. Accumulated ceramide per se does not seem to promote adhesion, since our experiments demonstrate that the adhesion of *P. aeruginosa* strains to washed nasal epithelial cells cultures from CF patients and control individuals did not differ (unpublished data).

Furthermore, it is conceivable that the accumulation of ceramide might severely impact the function of membrane rafts that were shown to be required for cell activation upon *P. aeruginosa* lung infections[55]. Curiously, hypersusceptibility to infection may also be induced by Peptamen^{R} feeding of CF-mice. While ceramide selectively displaces cholesterol from ordered lipid rafts,[56] high cholesterol levels may turn this reaction in the other direction, resulting in ceramide-poor cholesterol-rich rafts. Indeed, Guibault et al.[33] reported on a significant improvement of *Cftr*-deficient mice to combat *P. aeruginosa* infection when ceramide levels were normalized by treatment with Fenretinide.

Besides cell death, ceramide is also critically involved in the regulation of transcription factors[20], which could subsequently lead to the release of cytokines and

induce inflammation. The notion that the increased synthesis and release of cytokines in lungs of uninfected CF-mice, and the subsequent, age-dependent recruitment of effector cells is triggered by accumulated ceramide, is supported by the experiments revealing that partial Asm inhibition by amitriptyline or Asm heterozygosity abrogates both macrophage and neutrophil accumulation in the submucosa. How the accumulation of macrophages and neutrophils around submucosal glands contributes to the hypersusceptibility of CF-mice to *P. aeruginosa* lung infection remains to be defined. Both cell types have been implied in tissue remodeling mediated by the production of reactive oxygen species, serine and metallo-proteases[57,58] and their continuous exposure may affect submucosal gland structure and antimicrobial, anti-inflammatory and anti-oxidant gland function and finally provoke fibrosis and emphysema[57,58].

As direct or indirect consequences of the increased ceramide concentrations in cells of the respiratory tract, our results unify several previously published observations in CF mouse strains with regard to inflammation[10], apoptosis[52,53] and hypersusceptibility to *P. aeruginosa* infection.[30-32] Furthermore, our findings emphasize that older animals are hypersusceptible to severe pulmonary disease, an observation in accordance with previous studies by Pier and colleagues[29].

Our results strongly suggest that increased ceramide concentrations are deleterious for CF patients and that reduction of such levels by amitriptyline might be beneficial. However, it is important to note that ceramide concentrations should not be reduced under a critical cellular level by a future drug to treat CF, which would impair the biological functions of ceramide. For example, we have previously shown that mice completely lacking Asm (*Smpd1*$^{-/-}$) fail to adequately respond to acute infection with *P.*

aeruginosa.[21] These mice are unable to clear bacteria from airways by epithelial cell internalization via ceramide-dependent rafts and subsequent degradation and also fail to control the infection[21]. Previous studies demonstrated that 5-15% residual activity of the Asm were sufficient to permit a normal response of epithelial cells to *P. aeruginosa* infection. Therefore, we employed in the present study mice heterozygous for the Asm. Thus, Asm-heterozygosity and treatment with amitriptyline in doses used here do not affect the ability of epithelial cells to adequately react to acute *P. aeruginosa* infections, but they are sufficient to normalize ceramide levels in CF-mice. Consequently, a future drug, targeting ceramide to treat CF, should be carefully titrated to normalize ceramide levels in CF-lungs. A complete suppression of ceramide would paralyse early defense mechanisms of the host. This situation is reminiscent for many highly effective drugs, for instance anti-coagulants: Given at doses that moderately suppress coagulation, these drugs may prevent heart and brain infarcts, while doses that result in a stronger blockade of coagulation may result in life threatening bleeding.

In CF patients expressing normal levels of Asm, the described pathophysiological events may provoke a vicious cycle, since *P. aeruginosa* triggers Asm activation, and, hence, ceramide release leading to apoptosis[21]. Thus, the accumulation of ceramide on the respiratory epithelium, caused by the basic defect in CF, may still increase after the onset of chronic *P. aeruginosa* infection. In such a scenario, the increasing deposition of DNA (as a consequence of increased apoptosis) would further facilitate bacterial adhesion, impair mucociliary clearance by increasing the viscoelasticity of the airway surface liquid, and augment tissue destruction and remodeling by increasing inflammation.

In summary, our data identify ceramide as one of the key regulators of inflammation and subsequent infection in CF airways. Normalization of ceramide levels by amitriptyline represents a novel strategy to prevent bacterial infections in CF patients.

Methods

CF patients and healthy individuals

Ciliated epithelial cells were obtained from 18 CF patients and 17 controls (mean age 27 ± 7.8 years and 33 ± 6.5, respectively) from the University Clinic Tuebingen, Germany. All samples were obtained with permission of the ethic committee of the University of Tuebingen, Tuebingen, Germany. Samples of normal lungs from three succumbed donors (mean age 26 years) in which the contralateral lung was not utilized for transplantation, and samples from the explanted lungs from three CF patients receiving a double lung transplant, were obtained from the Dept. of Pathology, University of Southern California, in compliance with the University of Southern California Institutional Review Board, Los Angeles, USA. Tissue blocks of approximately 1 cm^3 from bronchi were cut and placed in 10% paraformaldehyde, freshly prepared in phosphate buffered saline (PBS), pH 7.4, stored at 4°C and paraffin-embedded.

Mice and treatments

For various treatment regimens, infection protocols and lung tissue investigations, different *Cftr* mutant mouse strains and their respective littermates were used. First, $Cftr^{tm1Unc}$-$Tg^{(FABPCFTR)}$ (abbreviated $Cftr^{KO}$) Jaw mice were originally obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and bred and housed in the vivarium of the University of Duisburg-Essen, Germany. These mice are genetically deficient for the murine equivalent to human *CFTR* (*Cftr*), but express human CFTR in the gut under control of a fatty acid binding protein (FABP) promoter, which prevents acute intestinal

obstruction[59,60]. These mice are on a mixed background consisting of C57BL/6, FVB/N and 129. Second, B6.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$ (abbreviated *Cftr*^{MHH}) congenic mice were used[61]. The inbred CF strain CF/3-*Cftr*$^{TgH(neoim)Hgu}$ was established by strict brother-sister mating from the original C*ftr*$^{TgH(neoim)Hgu}$ mutant mouse that produces low levels of Cftr, and was generated using insertional mutagenesis in the *Cftr* exon 10. Then, the congenic *Cftr*^{MHH} strain was generated by backcrossing the targeted mutation into the B6 inbred background[62]. Syngenic B6 mice were used as controls. *Cftr*^{MHH} mice were housed both in the Central Laboratory Animal Facility of the Medizinische Hochschule Hannover and the University of Duisburg-Essen, Germany. Third, 12 week old C57BL/6 congenic B6.129P2-*Cftr*^{tm1Unc} mice bearing the S489X mutation in *Cftr* (*Cftr*^{S489X}) were used; *Cftr*$^{+/+}$ were used as wild type littermate controls. *Cftr*^{S489X} mice were housed and maintained in the vivarium at Case Western Reserve University, Cleveland, USA[32]. Fourth, *Cftr*^{KO} mice were bred with *Smpd1*$^{-/-}$ mice that are on a C57BL/6 background. To obtain *Cftr*^{KO}/*Smpd1*$^{+/-}$ mice, the heterozygous offspring was backcrossed to *Cftr*^{KO} mice for at least 5 generations. Control mice used were C57BL/6 mice that are syngenic to *Cftr*^{MHH} and *Cftr*^{S489X} as well as C3H mice, which were obtained from Charles River or the local breeding colony of the central animal facility of the Medical School Essen.

The different laboratories, housing the mice in isolator cages, provided a pathogen-free environment. The mice were repeatedly investigated for microbial infections by bacterial culturing and serology and remained negative up to date. The hygienic status was repeatedly tested by a panel of common murine pathogens according to the FELASA recommendations of 2002[63].

All procedures performed on mice were approved by the Animal Care and Use Committee of the Bezirksregierung Duesseldorf, Duesseldorf, Germany

Amitriptyline was dissolved in distilled H_2O and injected i.p. at a dose of 10 mg/kg twice daily for 2.5 days. zVAD was injected i.p. at a dose of 8 mg/kg twice daily for 2.5 days.

If indicated mice were fed with Peptamen^R ad libitum. A single mouse consumed approximately 17-20 ml of the liquid diet daily, which is in accordance with previous reports[45].

Asm activity

Lungs were homogenized in a buffer consisting of 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA and 1% NP40 employing a dounce homogenisator followed by 3 rounds of sonication (Ultrasonic Processor tip sonicator). An aliquot of the samples was diluted to 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA and 0.1% NP40 and incubated with 0.05 μCi per sample [^{14}C]sphingomyelin (52 mCi/mmol; MP Biomedicals, Irvine, CA, USA), which was dried, resuspended in 250 mM sodium acetate (pH 4.5, 5.0 or 5.9), 1.3 mM EDTA, and 0.1% NP40 and bath sonicated for 10 min prior to addition of the substrate to the samples. The samples were incubated for 30 min at 37°C. The enzymatic reaction was terminated by extraction in 4 volumes of $CHCl_3$:CH_3OH (2:1, v/v), the samples were centrifuged and an aliquot of the upper aqueous phase was scintillation counted to determine the release of [^{14}C]phosphorylcholine from [^{14}C]sphingomyelin.

Acid ceramidase activity

Cells were lysed in 200 mM citrate/phosphate buffer (pH 4.5, 5.0 or 5.9), 300 mM NaCl, and 1% Triton X-100 on ice for 15 min. The lysates were diluted to 0.1% Triton X-100 and the substrate [^{14}]C_{16}-ceramide (0.1 µCi/sample, 55 mCi/mmol, ARC) was added. Prior to addition, the substrate was dried, resuspended in 150 mM NaCl, 0.05% Triton X-100 and sonicated to get micelles. The enzyme reaction was performed for 30 min at 37°C. The reaction was terminated by addition of 20 volumes ethanol, the samples were centrifuged, the supernatants dried, resuspended in $CHCl_3$:CH_3OH (1:1, v/v) and lipids were separated on TLC employing $CHCl_3$:CH_3OH:28%ammonium hydroxide (90:20:0.5, v/v/v). The TLC plates were exposed to X-ray films, the substrate and the product were identified by co-migration with standards, removed from the plate and liquid scintillation counted.

To determine the reverse Ac activity, the enzyme reaction was performed with 200 µM [^{14}C] lauric acid (50 mCi/mmol) and sphingosine (50 nmol/reaction) at pH 4.5, 5.0 and 5.9. Ceramide was identified by TLC and quantified as above.

Ceramide measurements

The lungs were removed, transferred into 1 ml $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v) and homogenized 30-times in a Dounce homogenisator and 3-times for 20 seconds each in a sonication bath. After addition of 200 µl H_2O, the samples were centrifuged for 5 min at 14,000 rpm. Serum samples were directly added to 1 ml $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v). The lower phase was collected, dried and subjected to alkaline hydrolysis of diacylglycerol in 0.1 N methanolic KOH at 37°C for 60 min. The samples

were re-extracted, the lower phase dried and resuspended in 20 µl of a detergent solution (7.5 % (w/v) n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylene-triaminepentaacetic acid). After sonication (10 min), the samples were added to 70 µl of a reaction mixture, containing 10 µl diacylglycerol kinase (GE Healthcare Europe, München, Germany), 0.1 M imidazole/HCl (pH 6.6), 0.2 mM diethylenetriamine-pentaacetic acid (pH 6.6), 70 mM NaCl, 17 mM $MgCl_2$ and 1.4 mM EGTA, 2 mM DTT, 1 µM ATP and 10 µCi $[^{32}P]\gamma$ATP. The kinase reaction was performed for 30 min at room temperature and terminated by addition of 1 ml $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v), 170 µl buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.2) and 30 µl of a 100 mM EDTA-solution. The samples were vortexed and the phases separated. The lower phase was collected, dried, dissolved in 20 µl $CHCl_3$:CH_3OH (1:1, v/v) and separated on Silica G60 TLC plates employing $CHCl_3$:CH_3OH:CH_3COOH (65:15:5, v/v/v). The TLC-plates were exposed to x-ray films and ceramide spots were identified by co-migration with a C_{16}-ceramide standard. Incorporation of $[^{32}P]$ into ceramide was quantified by liquid scintillation counting after removal of the spots from the plates. Ceramide amounts were determined by comparison with a standard curve using C_{16}-ceramide as substrate.

Cholesterol measurements

Cholesterol was determined using a kit (ECCH-100) from BioAssay Systems following exactly the protocol of the vendor. Briefly, cholesterol esters are converted to cholesterol, cholesterol is then metabolized to cholest-4-ene-3-one and NAD reduced to NADH by

cholesterol dehydrogenase. Absorbance at 340 nm was determined to measure cholesterol concentrations.

Isolation of cellular vesicles

To obtain cellular vesicles lung tissue was mechanically homogenized and incubated for 30 min at 4°C in 0.3 M sucrose, 10 mM TES (pH 7.4), and 0.5 mM EGTA to swell the cells. Cells were disintegrated by 40x Dounce-homogenization, nuclei and unbroken cells were pelleted by centrifugation for 5 min at 600xg and 4°C, the vesicles were permeabilized by 5 min incubation with 0.05% Triton X-100, washed and stained with anti-ceramide antibodies (1:100) for 45 min at 4°C. Vesicles were pelleted by centrifugation at 55000xg and stained with Cy3-coupled anti-mouse IgM antibodies. Vesicles were then analysed by FACS.

To investigate the role of the pH for ceramide accumulation, vesicles were isolated as above and intact vesicles were incubated in 50 mM PIPES-KOH, 40 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 μg/ml each aprotinin and leupeptin, 2 mM ATP, 10 mM phosphocreatine, 5 mM succinate, 50 μg/ml creatine kinase and 1% fetal calf serum at a pH of 4.5 or 5.9 and incubated at 37°C for 8 hrs. The vesicles were then analysed for ceramide by the DAG kinase assay as above. Alternatively, they were fixed for 15 min with 2% paraformaldehyde, washed, permeabilized with 0.05% Triton X-100, washed, blocked and stained with Cy3-coupled anti-ceramide antibodies as above and analysed by FACS.

Inhalation and pulmonary lavage

Recombinant human DNase (Pulmozyme^{R}) was diluted in 0.9% NaCl solution to 25 μg/ml and 1 ml H_2O was applied by inhalation for 20 min. Children receive generally an absolute amount of 2.5 mg DNase, while we applied 25 μg to the mice (100-fold lower). Taking into account that the smaller breathing tidal volume may result in a much smaller dose delivered, we employed an approximately 16-fold higher dose for mice than would have been calculated on a weight base (25 g for a mouse and 40 kg for a child, 1600-fold higher). The higher dose was also used to achieve maximum DNA digestion in the bronchi in our acute experiments and was established in pilot experiments measuring the effects of DNase doses of 2.5 μg/ml, 25 μg/ml, 100 μg/ml and 1 mg/ml on the infection with *P. aeruginosa* (n = 3 each). The optimal dose in these experiments was 25 μg/ml and, therefore, used for all experiments. The DNase was diluted to achieve complete inhalation within 120 min (this time was used to compromise between concentration of the DNase and inhalation time).

Mice inhaled 1 ml of the indicated inhibitors. Drugs were dissolved in 0.9% NaCl and inhaled for approximately 20 min. The drugs were Bafilomycin (1 μM), Nigericin (2.5 μM), NH_4Cl (25 mM), chloroquine (10 μM), n-oleoylethanolamine (100 μM).

The concentration of the drugs was determined in pilot experiments to achieve sufficient alkalinisation of vesicles in lung cells and, at the same time, to be without obvious side effects as for instance an increase of breathing frequency.

Inhalation was performed with a Pariboy SX nebulizer apparatus (Pari, Starnberg, Germany, Cat. # 085G3000). The fluid reservoir in this nebulizer is connected to an air

jet, which produces a fine aerosol that the mice inhaled via a mask. The mask consisted of an oral inhalation device for children (LL-Nebulizer, cat. # 012G6202). It was clipped at the sides to cover only the nose and the surrounding part of the face of the mice. The nose of a mouse was manually pressed into the opening of the mask for 15 min, which permitted an excellent control of the inhalation process. We used single mice in the aerosol experiments.

Pulmonary lavage was performed to obtain murine lung macrophages. To this end, the mice were killed and the trachea canuled with a plastic catheter (at least 5 mm inner diameter) connected to a syringe. The canule was fixed using surgical thread. The thorax was very carefully opened without injuring the lung. The lungs were filled with 1 ml 0.9% NaCl solution via the canule and the lavage fluid was very carefully collected by slow suction. The lungs were washed six times in total with each 1 ml of fresh 0.9% NaCl solution.

pH measurements

Isolated respiratory epithelial cells from CF- and wild-type mice were incubated with 70 nM lysosensor-green D189 (Molecular Probes) for 15 min and dye emission immediately analyzed by fluorescence microscopy. To isolate intact epithelial cells, large bronchi and the trachea were removed, and cells were gently removed from the tissue surface. To determine the pH, the fluorescence intensity in vesicles was determined by measuring the fluorescence intensity in areas of 1.5x1.5 mm. Samples were located with minimum light exposure. Images were captured with excitation at 488 nm employing an argon laser and were recorded at 505 nm emission. The measured fluorescence intensity

was compared with a standard curve. To obtain the standard curve, cells were permeabilized and resuspended in H/S with a given pH of 4.5, 4.7, 4.9, 5.1, 5.3, 5.5, 5.7, 5.9, 6.1, 6.3 and 6.5, incubated for 15 min with 70 nM lysosensor-green and the fluorescence intensity in an area of 1.5x1.5 mm was determined. The standard curve (random fluorescence units) then served to determine fluorescence intensity and, therefore, the vesicular pH in intact cells. If indicated intact cells were incubated with 10 μM of the CFTR inhibitor $CFTR_{inh}$-172 (Sigma) for 15 min prior to the addition of lysosensor-green. The inhibitor was present throughout the whole experiment. Since $CFTR_{inh}$-172 is very sensitive to storage in stock DMSO solutions at low temperature for prolonged periods of time, we used it immediately after solubilisation.

Fluorescence, confocal and electron microscopy, TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling), and DNA staining of lung sections

TUNEL: Lungs were fixed in 4% buffered PFA (pH 7.3) for 36 h and embedded in paraffin. The samples were deparaffinized, re-hydrated, treated with 0.1 M citrate buffer (pH 6.0) at 350 W for 5 min in a microwave, washed in PBS, blocked in 0.1 M TrisHCl (pH 7.5), 3% BSA and 20% FCS and incubated with fluorescein-coupled dUTP and TUNEL enzyme (Roche Diagnostics) in the presence of terminal deoxynucleotidyl-transferase. The samples were washed again with PBS, incubated for 10 min at 70°C to remove unspecific binding and labelled with an alkaline phosphatase-coupled anti-FITC antibody in PBS. The signal was then visualized by a colorimetric reaction, the sections were mounted in moviol and analyzed by light microscopy.

To visualize ceramide in epithelial cells, large bronchi were isolated from the indicated mice, fixed in 2% paraformaldehyde (PFA, pH 7.4) in PBS for 30 min and ciliated epithelial cells were scratched from the tissue. The cells were blocked in PBS supplemented with 5% fetal calf serum (FCS) and stained with monoclonal anti-ceramide antibodies (1:100 dilution, clone 15B4 from Alexis or clone MAS 0010 from Glycobiotech), followed by incubation with a secondary Cy3-labelled anti-mouse IgM antibody. The cells were analysed on a Leica fluorescence microscope DMIRE2 with an exposure time of 150 ms. All microscopy studies were done at 1000x magnification if not otherwise noted.

Human epithelial cells were collected by nasal brushings using cotton tips. The cells were then carefully washed 5-times in HEPES/Saline (H/S; 132 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$), fixed in 4% paraformaldehyde for 15 min and stained as above. The fluorescence signal was quantified in areas of 0.5 x 0.5 cm of the fluorescence pictures using the QFluoro software from Leica.

Ceramide in murine lung sections: Ceramide was stained on lung sections after fixation, embedding in paraffin, dewaxing and rehydration by incubation of the slides in PBS supplemented with 0.01% Tween 20 for 15 min. The slides were washed in PBS and the tissue was incubated with anti-ceramide-antibodies (1:100 dilution, clone MAS 0010) for 45 min at room temperature. The slides were washed 3-times in PBS supplemented with 0.01% Tween 20 and stained with a Cy3-coupled anti-mouse IgM secondary antibody (1:1000) for 45 min at room temperature. The sections were mounted in moviol

and analyzed by fluorescence microscopy as above or by confocal microscopy employing a Leica confocal microscope DMIRE 2.

Ceramide in human lung sections: Cryostat thin sections (5 μm) were prepared from lung tissues of CF-patients and normal individuals. Sections were fixed on glass slides with acetone, washed with PBS-Tween 0.1% and pre-incubated with normal goat serum, 1:10 diluted in PBS-Tween (0.1%). The sections were incubated with a 1:20 dilution of monoclonal antibodies to ceramide (Axxora, Grünberg, Germany) in PBS at room temperature for 2 h. Sections were washed with PBS/0.1% Tween and incubated with Cy2-labeled goat anti-mouse antibodies for 1 h at room temperature, washed 3-times with PBS/0.1% Tween and incubated with 2 μg/ml of 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI; Roche Mannheim, Germany) in PBS/0.1% Tween for 5 min at room temperature. The sections were washed and embedded with fluorescence mounting medium.

Electron microscopy: Tissue was perfusion-fixed with 4% paraformaldehyd, cut into 1 mm^3 cubes and washed with PBS/sucrose. Tissue was dehydrated in a graded ethanol series and embedded in LR-White. Immunogold double staining was performed on ultrathin sections of 70 nm. After blocking for 1 h the sections were incubated with primary antibodies at 4°C overnight. Thereafter the sections were washed 4-times with PBS-Tween and incubated with secondary antibodies (anti-mouse IgM conjugated with 25 nm and anti-goat antibody IgG conjugated with 10 nm, each diluted 1:25) for 1 h. The samples were washed 4-times with TBS-Tween and finally with H_2O. The sections were poststained with uranyl acetate and investigated with a Zeiss EM 910 microscope (Zeiss, Jena, Germany). Image documentations were done with Kodak 4489 macro EM film.

Corrections of background illumination and contrast enhancement were done in ImageJ (Wayne Rusband, NIH).

For DNA staining the rehydrated sections were incubated for 5 min in 4 μg/ml ethidium bromide, washed in PBS, mounted in moviol and analyzed by fluorescence microscopy.

IL-1 and keratinocyte-derived chemokine (KC) measurements

Murine lungs in PBS were homogenized in a dounce homogenisator for 30 sec. Cytokine concentrations were determined by commercial ELISA assays, following the instructions of the manufacturer (R&D, Germany).

***P. aeruginosa* and *S. pneumoniae* lung infection**

P. aeruginosa strain 762 was obtained from a patient with urosepsis, strain 769 from a patient with pneumonia, the laboratory strain ATCC 14115 was obtained from ATCC (Bethesda, USA). Bacteria were grown on tryptic soy bean agar plates overnight, removed from the plate, resuspended in tryptic soy bean bouillon at an optical density of 0.225 and grown at 37^{0}C for 60 min with gentle shaking (120 rpm) to obtain bacteria in the early logarithmic growth phase. The bacteria were washed twice in RPMI-1640, supplemented with 10 mM HEPES (pH 7.4), and resuspended at a density of $1x10^8$ CFU/20 μl in H/S. Mice were intranasally infected with 10^7 or 10^8 CFU of *P. aeruginosa* strains 762, 769 and ATCC 14115 as indicated, employing a plastic-covered 30-gauge needle, which was inserted 2 mm into the nose. To avoid changes in mucociliary clearance a very short and light ether anesthesia was employed[64]. *P. aeruginosa* cell

numbers were determined in murine lungs 15, 30, 60 or 120 min after challenge. Lungs were removed, homogenised and lysed for 10 min in 5 mg/ml saponin to release intracellular bacteria. Dilutions of the homogenates were cultured on TSB plates in duplicates. Bacterial counts, determined after 18 h, were normalized for lung weight. Additionally, the survival of infected mice was determined for seven days after challenge. Control experiments reveal that 10 mg/l amitriptyline did not affect growth of the bacteria.

S. pneumoniae strain R6 was grown on sheep blood tryptic soy agar for 12 h, removed from the plate and grown in TSB for 1 h. The mice were intranasally infected with 10^7 CFU and the number of bacteria in the lungs was determined 3 h after infection as above. To reduce the variation associated with *in vivo* infectious experiments, we employed a standardized protocol, which minimizes most variables. The details of the protocol are given in the supplement.

Determination of neutrophil and macrophage numbers and submucosal glands in murine lung tissues.

Cryostat thin sections (5 μm) were prepared from lung tissue of uninfected CF-, wild type or $Cftr^{-/-}/Smpd1^{+/-}$ mice. For these experiments all three CF-mouse types ($Cftr^{KO}$, $Cftr^{MHH}$, $Cftr^{S489X}$) were used. Sections were fixed on glass slides with acetone, washed with PBS-Tween 0.2% and pre-incubated with normal goat serum, 1:10 diluted in PBS-Tween (0.2%). The sections were incubated with a 1:50 dilution of monoclonal antibodies to murine neutrophils or to macrophages (CD68) (Acris, Hiddenhausen, Germany) in PBS-0.5% Triton, overnight at 4°C. Sections were washed with PBS/0.1%

Tween and incubated with Cy2-labeled goat anti-mouse antibodies for 1 h at room temperature, washed 3-times with PBS/0.1% Tween and incubated with 2 μg/ml of DAPI (Roche, Mannheim Germany) in PBS/0.1% Tween at room temperature. The sections were washed and embedded with mounting medium. For quantitative determination of cell numbers, five sections per mouse lung were examined. From every section, 6 to 10 digitalized images were taken (magnification: 200x; area: 850x650 μm). Sections were analysed with an Axioplan microscope (Zeiss, Oberkochen, Germany) using Axiovision (Zeiss). Submucosal glands in murine lungs were determined by staining with the periodic acid Schiff (PAS) reagent.

Adhesion of *P. aeruginosa* to DNA

To determine *P. aeruginosa* adhesion to and growth on DNA, 180 μg DNA or H/S were distributed on an agarose carbohydrate matrix and incubated with a suspension of $5x10^4$ CFU/ml of *P. aeruginosa* for 48 h at 37°C. Thereafter, bacteria were counted and photographs were taken. Furthermore, A549 respiratory cells were pre-incubated with 10 μg denatured salmon sperm DNA or medium for 30 min at 37°C. Cells incubated with a suspension of 1x 10^7 CFU/ml of *P. aeruginosa* for 2 h at 37°C. The supernatant was removed, the cells fixed with 4% formaldehyde, and *P. aeruginosa* stained with a Cy3-coupled anti-*P. aeruginosa* antibody. Eukaryotic cells were visualized with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI; Roche, Mannheim, Germany). Note: single stranded sperm DNA does not stain with DAPI.

Uptake of sphingosine

Tracheae from wild type-, *Cftr*^{KO} and *Cftr*^{MHH} mice were removed, added into RPMI 1640, supplemented with 10 mM HEPES and 10% FCS, opened and incubated with 5 μCi [^{3}H]sphingosine (20 Ci/mmol, American Radiolabelled Chemicals) and 2 μM sphingosine or 5 μCi [^{14}C]sphingosine-1-phosphate (25 mCi/mmol, American Radiolabelled Chemicals) and 2 μM sphingosine-1-phosphate for 45 min at 37°C as described[15]. The tracheae were extensively washed in PBS and uptake was determined by liquid scintillation counting.

Statistics

Data are expressed as arithmetic means ± SD and statistical analysis was made as indicated. In the case that values were not normally distributed, exact Mann-Whitney tests were performed. For normal distributed values oneway ANOVA was applied. Significances are indicated. Kaplan-Meyer curves were analyzed by the log rank test ($p < 0.05$). All data are obtained from independent measurements.

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Legends

Fig. 1: Cftr-deficiency results in pulmonary ceramide accumulation

(**a**) CF-mice accumulate pulmonary ceramide. Ceramide concentrations increase with age in lung tissue of $Cftr^{KO}$ and $Cftr^{MHH}$ mice. Wild type mice were either C3H (wt) or C57BL/6 mice (wt (B6)), the latter are syngenic to $Cftr^{MHH}$ mice. Shown are the mean ± SD of 6 independent experiments performed with each one mouse/group. Asterisks indicate significant differences compared to the respective wt mice (ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

(**b-c**) Fluorescence microscopy of isolated epithelial cells (**b**) and paraffin sections from lungs (**c**) of $Cftr^{KO}$ and $Cftr^{MHH}$ mice with Cy3-coupled anti-ceramide antibodies reveals a massive accumulation of ceramide in respiratory epithelial cells and in the submucosa compared to C3H and C57BL/6 (B6) wild type mice. In contrast, no accumulation of ceramide was observed in alveolar epithelial cells (lower panels in **c**). Control stainings with irrelevant Cy3-coupled antibodies were negative (not shown). Cells and histology sections were analysed on a DMIRE2-Leica fluorescence microscope with an exposure time of 150 ms. Shown are representative results from 6 independent experiments. (Magnification 1000x, L = lumen of the bronchus, E = epithelial cells, S = submucosa).

(**d-f**) Confocal microscopy (**d**), FACS studies (**e**) and immunogold electron microscopy (**f**) reveal an accumulation of ceramide containing vesicles in *Cftr*-deficient epithelial cells. The FACS data also indicate an increased concentration of ceramide in *Cftr*-deficient vesicles as measured by the increased Cy3-signal in the ceramide-positive

vesicle population isolated from *Cftr*-deficient cells compared to vesicles from wild type cells. All confocal microscopy studies were done with the same microscopy settings on paraffin sections of 16 week-old wild type and $Cftr^{MHH}$ mice (Magnification 1000x). The sections were stained with Cy3-anti-ceramide antibodies. FACS studies were performed on crude vesicle preparations from lungs of the indicated mice. Vesicles were stained with Cy3-coupled ceramide antibodies. The electron microscopy studies were performed on 16 week-old wild type and $Cftr^{MHH}$ mice. The sections were stained with 25 nm gold coupled anti-ceramide and 10 nm gold coupled anti-cathepsin D antibodies. Analysis of sections from $Cftr^{KO}$ revealed the same results. The confocal and FACS studies are representative for 10 independent experiments with very similar results, the electron microscopy for 3 independent studies.

(**g-i**) Fluorescence microscopy analysis of nasal epithelial cells (**g**) or lung tissue (**h, i**) from CF patients or healthy individuals reveals accumulation of ceramide in the epithelial cells (**g, h**) and submucosal glands (**i**) compared to the respective cells or tissues from healthy individuals. Control stainings with irrelevant Cy3- or Cy2-coupled antibodies were negative (not shown). Cells were analysed on a DMIRE2-Leica fluorescence microscope with an exposure time of 140 ms (**g**) or a Zeiss Axioplan microscope at an exposure time of 800 ms (**h, i**). The arrows in panel i indicate submucosal glands. Shown are representative results from 18 CF and 17 healthy individuals (nasal respiratory cells) or 3 lung samples, respectively. In the lung samples we analysed a similar number of airways each in healthy and CF tissues. Original magnification: x 1000 in panel h and x 400 in panel i.

Fig. 2: The increase of vesicular pH in *Cftr*-deficient cells results in cellular ceramide accumulation, corrected by pharmacological or genetic inhibition of the Asm

(**a**) Staining of respiratory epithelial cells, isolated from CF- and control mice with 70 nM lysosensor-green for 15 min and immediate analysis by fluorescence microscopy reveals an increase of the vesicular pH in *Cftr*-deficient cells to approximately pH 5.9. As indicated wt cells were incubated with 10 μM of the CFTR-inhibitor $CFTR_{Inh}$-172. Shown is a typical analysis from 500 cells total (100 epithelial cells from 5 different mice) and the calculated pH in those vesicles after comparison with a standard curve (lower panels) obtained from the fluorescence signal at a given pH. The standard curve was obtained from cells that were permeabilized, stained with 70 nM lysosensor-green for 15 min, resuspended in H/S with pH values between 4.5 and 6.0 and the fluorescence signal was determined in an region of interest (1.5 mm x 1.5 mm).

(**b**) Increase of the pH from pH 4.5 to pH 6.0 results in a ~90% reduction of the activity of acid ceramidase (Ac) to cleave ceramide, while its activity to generate ceramide from sphingosine (reverse activity) increases during this pH shift. The activity of Asm to cleave sphingomyelin to ceramide is only reduced by 35% during the same pH shift.

(**c**) Alkalinization of intracellular vesicles of lung cells by inhalation of bafilomycin, nigericin, chloroquin or NH_4Cl results in an accumulation of ceramide in wild type mice lungs.

Panels **a-c** show the mean ± SD of 5 independent experiments with one mouse each. Asterisks indicate significant differences compared to wt (**a**) or untreated mice (**c**) (ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).

(**d**) Acidification of vesicles isolated from *Cftr*-deficient mice normalizes ceramide concentrations. Vice versa, alkalinization of wild type vesicles to pH 5.9 results in accumulation of ceramide within these vesicles. The upper panel shows ceramide levels determined by DAG-kinase assay from 5 independent studies with one mouse each (ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001). The FACS plots in the lower panel display the accumulation of ceramide in a large population of vesicles isolated from 24-week old $Cftr^{MHH}$ mice, which is corrected by acidification. The intensity of the staining with Cy3-coupled anti-ceramide antibodies served to measure the amount of ceramide in a single vesicle. We detect at least 3 populations of vesicles containing different amounts of ceramide. The population showing a high amount of ceramide at pH 5.9, which is reduced by acidification to pH 4.5, is indicated by an arrow. The FACS results are representative of 3 similar independent studies.

(**e-g**) Inhibition of Asm by i.p. injection of amitriptyline (Ami) (10 mg/kg twice daily for 2.5 days) or heterozygosity of Asm ($Cftr^{-/-}/Smpd1^{+/-}$) significantly reduces the activity of the Asm, the reverse and forward activity of the Ac at pH 5.9 (**e**). Amitriptyline injection or heterozygosity of the Asm almost normalize pulmonary ceramide levels (**f**, **g**). Ceramide was determined by DAG-kinase assays (**f**) or fluorescence microscopy after staining with Cy3-coupled anti-ceramide antibodies (**g**). Displayed are the mean ± SD or representative results of 6 independent experiments.

Asterisks * represent significant differences compared to wt (C3H or C57BL/6) mice or untreated mice or as indicated (ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).

Fig. 3: Ceramide-accumulation mediates hypersusceptibility of CF-mice to *P. aeruginosa* infections

(**a, b**) *Cftr*^{KO} and *Cftr*^{MHH} mice are hypersusceptible to *P. aeruginosa* infections and contain high numbers of *P. aeruginosa* in the lung 2 h after intranasal application of 10^8 CFU *P. aeruginosa* strain 762 (**a**), 769 (**b**) or 14115 (**b**), while wild-type mice (C3H or C57B/L6) mice, treated with amitriptyline, or Asm-heterozygotic mice (*Cftr*$^{-/-}$/ *Smpd1*$^{+/-}$) are resistant to infection. Demonstrated are the mean ± SD of 6 independent experiments. Asterisks * represent significant differences as indicated (exact Mann-Whitney tests, *p < 0.05, **p < 0.01, ***p < 0.002). Please note that the y-scale is logarithmic. The CFU/100 mg lung of 16 week old *Cftr*^{Ko} mice are (9.9 ± 1.9) x 10^5, of the 24 week old *Cftr*^{Ko} mice (11.6 ± 1.7) x 10^5. The CFU/100 mg lung for 16 week old Cftr^{MHH} mice are (8.5 ± 0.5) x 10^5 and for the 24 week old mice (9.7 ± 0.45) x 10^5.

(**c**) Immediately, i.e. 2 min after intranasal application of 10^8 CFU of planctonic, early log phase *P. aeruginosa* almost all bacteria were recovered from the noses of the mice (upper left panel). A detailed time course of the number of bacteria in the lung after intranasal infection reveals that pulmonary bacteria numbers do not differ significantly between wild-type and amitriptyline-treated or untreated *Cftr*^{KO} and *Cftr*^{MHH} 15, 30 and 60 min after challenge with 10^8 CFU of *P. aeruginosa* (lower panel). Wildtype and amitriptyline-treated *Cftr*^{KO} and *Cftr*^{MHH} mice then start to eliminate most bacteria within the following 60 min (i.e. up to 120 min after infection), while the bacteria

logarithmically grow in CF-mice. Infection of the mice with 10^7 CFU results in approximately 10-fold lower absolute numbers in the lung, but a very similar proportion between wt and CF-mice (upper right panel). Shown are the mean ± SD of 6 independent experiments, significant differences were determined using exact Mann-Whitney tests (*p < 0.05, **p < 0.01, ***p < 0.002).

(**d**) The mortality of *Cftr*^{KO} and *Cftr*^{MHH} mice after intranasal infection with 10^8 CFU *P. aeruginosa* 762 is increased compared to wild-type mice and prevented by pharmacological inhibition or heterozygosity of Asm. Wild-type mice treated with amitriptyline were as resistant as untreated wild-type mice (not shown). Displayed are Kaplan-Meyer curves, significant differences ($p < 0.05$) to controls were determined by the log-rank test and are indicated by *.

Fig. 4: CF-mice suffer from constitutive pulmonary inflammation corrected by normalization of pulmonary ceramide levels

(**a**) IL-1 and KC concentrations are constitutively increased in uninfected 24 week old *Cftr*^{KO} and *Cftr*^{MHH} mice. Pharmacological or genetic inhibition of Asm by amitriptyline or heterozygosity of Asm normalizes cytokine levels in the lung of CF-mice. Cytokine levels were measured by commercial ELISA in aliquots from lung homogenates. Shown are the mean ± SD of 6 independent studies. Significant differences are indicated by asterisks (ANOVA, *p < 0.05, **p < 0.01).

(**b**) Macrophage and (**c**) neutrophil cell numbers age-dependently increase in uninfected CF-mice. Lung tissue sections were labeled with specific monoclonal antibodies to macrophages and neutrophils and anti-rat Cy2-labeled goat antibodies. Cell

numbers were determined in digitalized images using Axioplan microscope with the Axiovision software. Pharmacological or genetic inhibition of Asm by amitriptyline or heterozygosity of Asm normalizes cell numbers in the lung of 30-week old CF-mice. Significance between age-matched CF mice and wild type mice or between untreated CF mice and amitriptyline-treated CF and *Asm/Smpd1*$^{+/-}$ mice, respectively (*$p < 0.05$, **$p < 0.01$) was determined using ANOVA.

(**d-m**) Macrophages (**d-i**) and neutrophils (**j-m**) accumulate around submucosal glands and in BALT in uninfected CF-mice (**d**, **f**), but not wild-type (**h, l**) mice. Macrophages (green) are indicated with an arrow. Submucosal glands (black arrow) (**e**), in the subsequent tissue section to that in panel **d**, were stained with the periodic acid Schiff (PAS) reagent. Panels **g**, **i**, **k and m** represent 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) stained tissue sections of **f, h, j and l**, respectively. Original magnification **d, e**: x400; **f-m**: x100.

Fig. 5: Pulmonary ceramide accumulation results in constitutive increase of respiratory cell death and deposition of DNA on the respiratory epithelium

(**a**, **b**) TUNEL stainings of lung sections reveal an increased death of respiratory epithelial cells, dispersed in the respiratory mucosa of large and medium-sized bronchi in *Cftr*^{KO} and *Cftr*^{MHH} mice. Pharmacological or genetic inhibition of the Asm or application of the broad-spectrum caspase inhibitor zVAD normalizes the death rate of respiratory epithelial in CF mice. Red staining of nuclei indicates dead cells in the TUNEL assays. Panel **a** shows representative TUNEL stainings of large bronchi, panel **b** gives the mean ± SD of the quantitative analysis of dead cells counted in at least 10 bronchi/mouse of 6

mice per group. Asterisks represent significant differences (ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).

(**c** and **d**) CF-mice display increased DNA-deposits (arrows) on the mucosal surface of bronchi, which are prevented by pharmacological or genetic inhibition of the Asm, treatment with the broad-spectrum caspase inhibitor zVAD or inhalation of DNase. (**c**) DNA was stained in paraffin sections either by TUNEL (upper row) or with ethidium bromide (lower rows) that intercalates into DNA resulting in a bright right staining of DNA. DNA-deposits are indicated by arrows, the epithelial cell layer is indicated by an E, the bronchial lumen by L. Shown are representative results from the analysis of at least 10 bronchi/mouse from 6 mice/group. Panel **d** shows the percentage of bronchi displaying one or more DNA-deposits (mean ± SD). Significant differences are indicated by asteriks (ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).

Fig. 6: DNA deposits are critically involved in the high susceptibility of CF-mice to *P. aeruginosa* infection

(**a, b**) DNA, distributed on agarose as a carbohydrate matrix (**a**) or on A549 respiratory cells (**b**), significantly increases adherence and growth of *P. aeruginosa*. Shown are representative photographs from 6 (**a**) or 3 (**b**) independent experiments. In panel (**b**) *P. aeruginosa* was stained with Cy3-coupled anti-*P. aeruginosa* antibodies, the cells were counterstained with DAPI.

(**c**) Treatment of $Cftr^{KO}$ and $Cftr^{MHH}$ mice with zVAD or recombinant human DNase prior to bacterial challenge prevents pulmonary infection with *P. aeruginosa* 762.

The caspase inhibitor was i.p. injected twice daily for 2.5 days prior to intranasal application of 10^8 CFU *P. aeruginosa*. DNase was applied by 20 min inhalation of 25 μg/ml DNase 60 min prior to infection with 10^8 CFU *P. aeruginosa*. Displayed are mean ± SD from 6 mice/group, studied in 6 independent experiments. Significant differences are indicated (ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).

Fig. 7: Very high cholesterol levels inhibit the Asm and normalize ceramide in CF-mice

(**a** and **b**) Long term feeding of $Cftr^{MHH}$ or $Cftr^{KO}$ with $Peptamen^R$ reduces ceramide (**a**) and increases cholesterol (**b**) concentrations in the lung, effects that are blocked by treatment with simvastatin (Sim). The mice were fed for 3 weeks with ad libitum $Peptamen^R$ and lung ceramide and cholesterol were determined. Asterisks indicate significant differences. Shown are the mean ± SD from each 5 independent experiments (ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001 for panel a and Mann-Whitney test for panel b, * p < 0.04).

(**c** and **d**) Increased cholesterol concentrations reduce the activity of the Asm in the lung after 14 day feeding with $Peptamen^R$ or incubation of isolated epithelial cells with increasing doses of cholesterol. Displayed are mean ± SD of 5 independent experiments each, significant differences are indicated by asterisks (exact Mann-Whitney tests, *p < 0.05, **p < 0.01, ***p < 0.002).